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Research Paper

Identification of *Phomopsis* Species on Some Ornamental and Forest Plants in Iran on the Basis of the Morphological and Molecular Characteristics

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Abstract

Phomopsis is a genus of imperfect plant pathogenic fungus whose hosts comprise several species in different regions of the world, such as grapes, soybean, acacia, hollyhock, velvetleaf, and several other plants. In this study, samples were collected from hollyhock, velvetleaf, purple bauhinia, and acacia plants suspected to be infected with *Phomopsis* fungi. They were, then, cultured and subjected to the separation and purification steps, and morphological characteristics of the derived isolates were determined. Among the isolates, five representatives (P1 to P5) were selected from Mazandaran province and their DNAs were extracted. The TEF and ITS regions of DNAs were amplified and sequenced. The ITS (4 and 5) region from ribosomal DNA and a part of the TEF gene were amplified and sequenced. The nucleotide sequence of the isolates was compared with the corresponding sequences available in the GeneBank, and the phylogenetic tree of the isolates was constructed. Results showed that the sequence of the isolates P1, P2, and P4 had the highest similarity with *Phomopsis malvacearum* species. The sequence of the isolate P3 was very similar to *Phomopsis loropetali*, and the sequence of the isolate P5 was very similar to *Phomopsis theicola*. The average size of pycnidia produced on three culture media, i.e. CDA, CMA, and PDA, was measured 16 days after incubation at 20-25°C. Sizes were 256 × 85 µm in acacia, 230 × 68 µm in velvetleaf, 230 × 69 µm in hollyhock and 193 × 47 µm in purple bauhinia isolates. Morphological and molecular features of the identified species were matched. The species identified here are reported from Iran for the first time. Symptoms of *Phomopsis* infection varied with host and identified species.

Keywords: Molecular identification, Morphological identification, *Phomopsis*.

INTRODUCTION

The imperfect fungi or Deuteromycetes belonging to the higher fungi commonly form no sexual reproductive structures, such as asci or basidia, which are produced by ascomycetes or basidiomycetes, respectively. Consequently, we frequently encounter their asexual forms (Barnett and Hunter, 1998). *Phomopsis* (Sacc.) Bubák (teleomorph: *Diaporthe*) is a fungal plant pathogen whose numerous species can infect various hosts, including grapes, soybean, hollyhock, velvetleaf, and several other plants in different regions of the world (Uecker, 1988). Two types of their spores are formed within *P. pycnidia*. The first type is α-conidia that are colourless and oval to spindle-shaped, while the other one is β-conidia, which are colourless and long, narrow, and arched. The presence of both types of conidia is required for accurate identification of *Phomopsis* (Uecker and Johnson, 1991; Boerema *et al*., 2004).

It is clear that the identification methods based on morphological features are unable to precisely distinguish different species of the favourite fungus because they are either commonly shared or extremely variable among the studied isolates. Currently, it has been indicated that the internal transcribed spacer (ITS) region of the fungus could potentially be used for the DNA barcode marker for fungi and also has the highest chance of successful identification of a wide range of examined fungus (Harrington *et al.,* 2000; Schoch *et al*., 2012). Moreover, combinations of morphological data with the molecular information, including the microsatellite-primed PCR (MSP-PCR) patterns and the sequencing of the rDNA-ITS region (ITS1–5.8S–ITS2), have contributed to identifying four new *Phomopsis* species (Santos and Philips, 2009; Santos *et al*., 2010; Hibbett and Taylor, 2013; Vedashree *et al*., 2015). In a previous research study by Mirabolfathi *et al*. (2013) on the branches of *Corylus avellana* in Iran, scabies composed of irregular wooden structures were observed. To identify of the ITS (ITS1-5.8S-ITS2) region of the pathogens, they used the amplification of Translation Elongation Factor 1-alpha (TEF) based on which the disease was diagnosed as *P. amygdali*. In Banihashemi and Javadi (2009), the cause of canker in fig branches in Fars province, Iran was reported to be the fungus *Phomopsis cinerascens*. This study was conducted to identify different species of the fungus *phomopsis* on the basis of morphological and molecular features on four host plants including acacia, velvetleaf, purple bauhinia, and hollyhock in Mazandaran province, Iran.

MATERIALS AND METHODS

Fungal isolation and purification

Samples were collected from the four plant species that were considered as the host of the fungus *Phomopsis* in Mazandaran province, Iran. These plant species included acacia (*Robinia pseudoacacia*), velvetleaf (*Abutilon theophrasti*), purple bauhinia (*Phanera purpurea*), and hollyhock (*Alcea rosea*). Stems and branches of fifty plants suspected to be infected with the fungus were transferred to a laboratory for the isolation and purification of the fungi. To isolate the fungus *Phomopsis* from the collected samples, the *Phomopsis* symptomatic area was disinfected with a piece of cotton soaked in 70% alcohol. The infected plant sections (flanked by the healthy and the infected tissue) were cut at a thickness of 4-5 mm and disinfected in a solution of 1% sodium hypochlorite (NaClO) for one minute. Then, they were immediately washed with distilled water three times. The disinfected plant tissues were dried on filter sterile paper and transferred to Petri dishes containing 1.5% water agar (WA) medium. The Petri dishes were kept at room temperature (25 °C) for 24-48 hours and the newly grown hyphae of the fungus were cultured in a new potato dextrose agar (PDA) medium. The samples were re-cultured by the hyphae tip. Then, they were transferred to the tubes containing the PDA medium, and the tubes were kept in a refrigerator.

Pathogenicity tests

Pathogenicity tests were done on the selected isolates. So, the isolates were inoculated on the branches of the plants. Stems and branches with a diameter of 0.5-1 cm were removed from healthy plants and transferred to the laboratory. Parts of the branches, 10–20 cm long, were selected and superficially disinfected with 0.5% NaOCl for three minutes and rinsed with distilled water. Then, they were dried with sterile towels. To prevent water loss, both ends of the samples were immersed in molten paraffin (70°C). In the middle of the branch, one part of the skin was sterilized again with 75% ethanol, and three cuts at a size about three sides of the square (one cm) were created in the skin. A piece of 6-8 mm with a thickness of 2 mm was removed from the young culture of the isolates and placed under the skin and the inoculation place was closed with parafilm. The control sample was inoculated only with the agar block. The inoculated branches were kept in a sterilized glass containing wet sponge at 20-25°C for 10-15 days until the symptoms appeared. After the development of the disease, pathogenic fungi were isolated again from the artificially infected area (Vrandecic *et al*., 2004; Jurkovic *et al*., 2007; Banihashemi and Javadi, 2009; Udayanga *et al*., 2014).

Morphological identification

For morphological identification, the isolates were cultured in a nutrient medium and their growth, formation of pycnidia, sexual fruiting body formation, the shape of the colonies, and other morphological characteristics were studied. The used media were Czapek Dox Agar (CDA), Corn Meal Agar (CMA), and Potato Dextrose Agar (PDA) (Farr *et al*., 2002; Udayanga *et al*., 2014; Annesi *et al*., 2016). The CDA medium, containing different elements in its composition, is used as a rich and ideal medium for a lot of fungi. The present study used it to identify the isolates of *Phomopsis*. A piece of the original strain grown in the culture medium was removed by a sterilized blade, and it was placed at the centre of Petri dishes containing any of the mentioned media. After seven days of shooting (Fig. 1), to measure the size of pycnidia, 50 pycnidia of each isolate were randomly selected, and the maximum length and width were measured with a calibrated microscope (Nikon model eclipse E600) lens. The average of the measurements was recorded as the pycnidia of each strain (Fig. 2).

Molecular identification

For molecular identification, the universal primers of ITS and TEF (Table 1) were used. Based on the morphological characteristics obtained, five isolates were selected and their genomic DNA was extracted by the modified procedure of Doyle and Doyle (1990). To sequence the DNA of the samples, MEGA 5.05 software was used and the neighbour-joining method was used to analyze the results (Tamura *et al*., 2013). Using blast search and articles related to the taxonomy of coelomycetes fungi, the appropriate outgroup was selected. The sequences obtained by using Bio Edit ver. 7.0.9.0 software were aligned and compared with the sequences available in the GeneBank. According to the highest similarity, the molecular identification of the isolates was performed and the phylogenetic tree was drawn using two methods of neighbour-joining and maximum likelihood with 1,000 bootstraps (Gomes *et al*., 2013; Udayanga *et al*., 2012).

RESULTS

Acacia, velvetleaf, hollyhock, and purple bauhinia having canker symptoms were obtained and the fungal colonies were grown on the PDA medium.

Morphological identification

The isolates grown on three media of CDA, CMA, and PDA were measured as described below.

Table 1. Names and sequences of primers used in this study and their source, temperature, time, and the number of the PCR cycles and amplified fragment sizes (bp).

Locus	Name and sequencing of primers (5' … à ⟶ 3')	Temperature and time on the PCR cycle	Size (bp)	Reference
ITS	ITS4: TCCTCCGCTTATTGATATGC	(94°C: 60 s, 58°C: 60 s, 72°C: 1 min) 10, 35 cycles	580	White *et al*., 1990
	ITS5: GGAAGTAAAAGTCGTAACAAGG			
TEF 1-α	EF1-1567R: ACHGTRCCRATACCACCRATCTT	(94°C: 60 s, 54°C: 60 s, 72°C: 1 min) 10, 35 cycles	370	Rehner and Buckley, 2005
	Efdf: AAGGAYGGNCARACYCGNGARCAYGC			

Czapek Dox Agar Medium (CDA): The colours of colonies were white to grey on the CDA medium. The isolates covered the surface of the medium with mycelium 7 days after incubation (Fig. 1). The colour and the growth of the colonies and fruiting body formation in CDA were similar to the PDA media, but there were minor differences in pycnidia size. Fruiting bodies (pycnidia) were produced after 12–16 days. The pycnidia ranged in colour from dark brown to black. The pycnidia form was oval-shaped in the isolates of acacia. In terms of the size of the isolates of velvetleaf and hollyhock, they were fusiform and very close to each other in terms of shape and size. Pycnidia on the isolates of purple bauhinia were almost circular and smaller than other isolates in terms of size. The average size of pycnidia produced in the CDA medium after 16 days was 262 × 91 µm in acacia isolates, 240 × 72 µm in velvetleaf, 223 × 68 µm in hollyhock, and 184 × 45 µm in purple bauhinia.

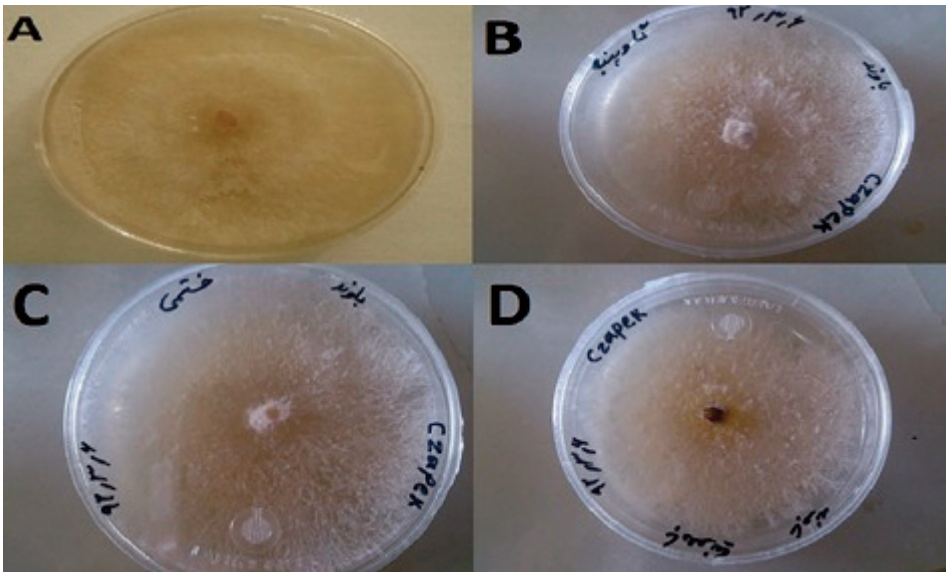

Fig. 1. The *Phomopsis* isolate colonies on the CDA medium after seven days; A) acacia isolate, B) velvetleaf isolate, C) hollyhock isolate, and D) purple bauhinia isolate.

Corn Meal Agar Medium (CMA): The colour of the colonies was also white to grey on the CMA medium. The growth of the isolates in the CMA medium was natural and almost the same with other growing media in the early days after cultivating, but then the growth stopped. Among the isolates, the growth of the purple bauhinia isolate had been much weaker than the rest since the first day of culture. Their hyphae also had low-density masses. The colony growth of all isolates in the CMA medium stopped after 14 days and pycnidia were not produced.

Potato Dextrose Agar Medium (PDA): The colour of the colonies was greyish-white on the PDA medium. The size and shape of pycnidia in the PDA and CDA media were similar. Masses of hyphae in all isolates had a high density, and after 12–16 days, a large number of pycnidia were produced. The colour of pycnidia ranged from dark brown to black (Fig. 2). After 16 days, the average size of pycnidia produced in the PDA medium was 250 × 80 µm in acacia isolates, 220 × 64 µm in velvetleaf, 237 × 70 µm in hollyhock, and 202 × 48 µm in purple bauhinia. The morphology of the studied isolates showed high similarity to the form genus *Phomopsis,* and this similarity was consistent with molecular analysis.

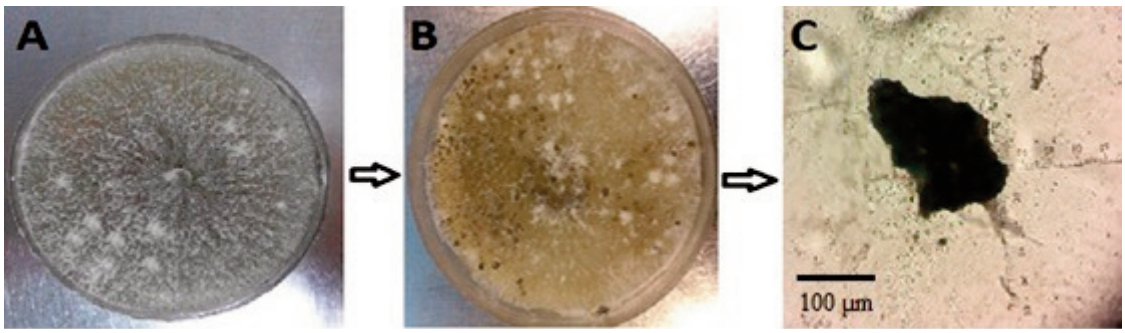

Fig. 2. A) The *Phomopsis* colony on the PDA medium after 7 days, B) pycnidia production after 12-16 days, C) measuring the size of pycnidia.

Molecular identification of isolates

The results of the pathogenicity test showed that *Phomopsis* spp. is the cause of canker on acacia, velvetleaf, hollyhock, and purple bauhinia. A total of 50 purified fungal isolates were obtained in which five isolates were selected for molecular identification as follows: P1 sample from hollyhock, P2 from velvetleaf, P3 from purple bauhinia, and P4 and P5 from acacia.

The desired ITS genomic region of the represented isolates P1, P2, P3, P4, and P5 were amplified (Fig. 3). The length of the amplified fragment was estimated to be nearly 580 bp (compared to the molecular weight marker on the agarose gel). The obtained sequences were compared with other corresponding sequences available in the GeneBank using BLAST software in the NCBI database. The phylogenetic tree was drawn on the basis of the ITS sequence of the isolates, and some sequences in the GeneBank using two different methods including NJ and ML with 1,000 bootstraps (Fig. 4). The sequences of isolates P1, P2, and P4 showed the most similarity with *Phomopsis malvacearum* species. The sequences of the isolate P3 showed the most similarity with the species *Phomopsis loropetali,* while the sequences of the isolate P5 showed the most similarity with *Phomopsis theicola* species.

The sequence of the TEF-1α gene of the representative *Phomopsis* isolates from four host plants was amplified, and then, they were searched for the similarity by BLAST software in the NCBI database of GeneBank. The length of the amplified fragment was estimated to be nearly 370 bp (compared to the molecular weight marker on the agarose gel). Owing to the small number of this gene related to the genus *Phomopsis*, an acceptable result was not revealed regarding the application of blasts in GeneBank. However, the comparison of sequences to each other showed that the sequences of isolates P1, P2, and P4 had the highest similarity together and with *P. malvacearum* species, which was consistent with the results of the ITS gene.

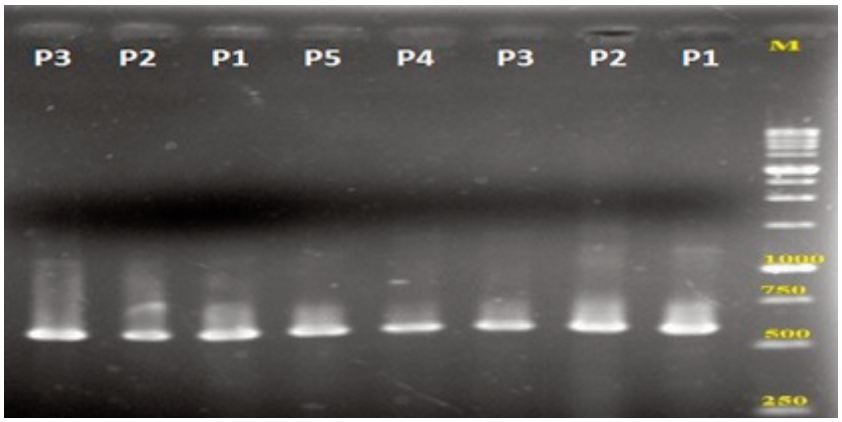

Fig. 3. Amplified fragment of ITS gene for *Phomopsis* isolates P1, P2, P3, P4, and P5 on 1.2% agarose gel; M: shows the DNA molecular weight marker with 1 kb of Fermentas, Lithuania.

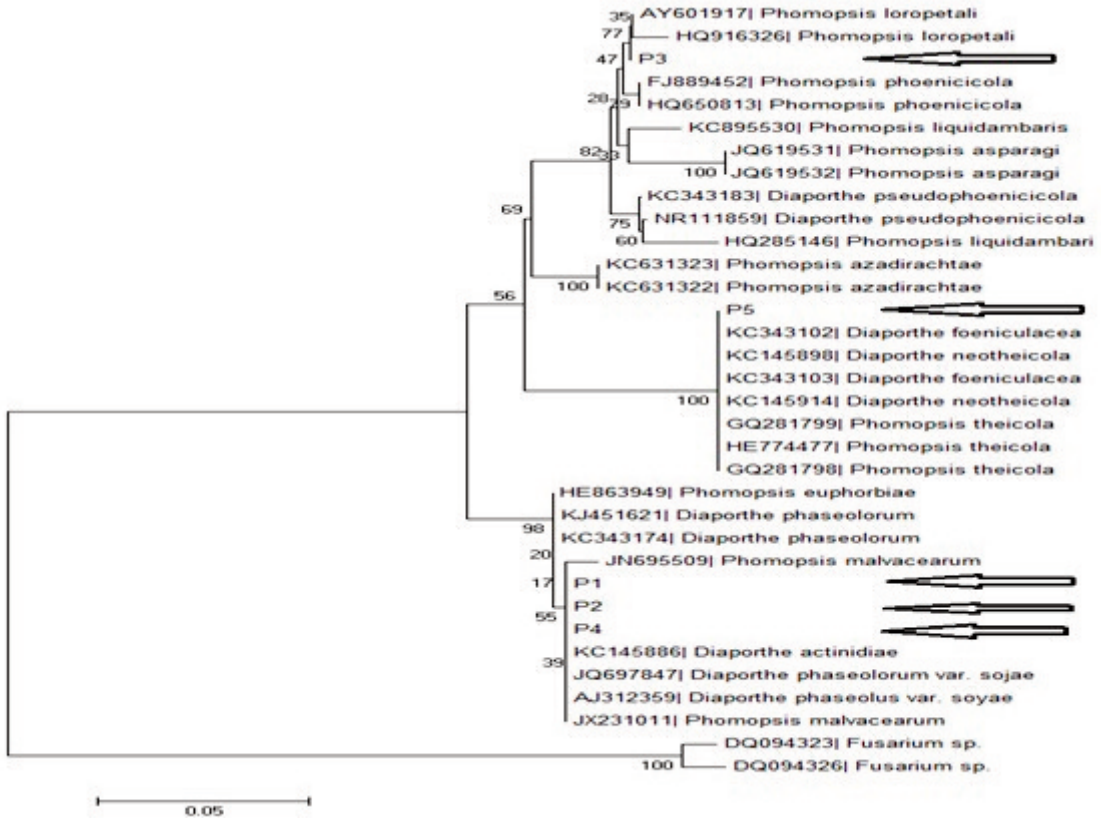

Fig. 4. The phylogenetic tree inferred by the sequence of ITS isolates P1, P2, P3, P4, and P5 obtained from the four host plants in Mazandaran, Iran and other isolates registered in the GeneBank by the neighbour-joining method and with MEGA5 programme; the numbers recorded in the place of branches show clustering percentage out of 1,000 repeated biopsies (Bootstrap).

Symptoms of pathogenicity of species

Morphological and molecular studies indicated a high similarity of the studied isolates with the sub-genus *Phomopsis*. Among the isolates of hollyhock, velvetleaf, and one isolate of acacia were detected the species of *Phomopsis malvacearum*. Also, among the isolates on purple bauhinia, the species of *P. loropetali*, and among the isolates on acacia, the species of *P. theicola* were iden-

tified. The symptoms of the disease caused by the fungus studied on four different hosts were investigated. The signs of fungus *Phomopsis* infection on velvetleaf and hollyhock were similar regarding the form of elliptical spots, stretched, and with dark colours. After the spread of the disease, a grey halo of mycelium of fungus formed on the patch. In some cases, the disease spots were linked to each other and created larger spots (Fig. 5). These home-like symptoms on both morphological and molecular characteristics of both hosts matched and all strains of the species *P. malvacearum* were identified.

The early symptoms of *Phomopsis* on acacia were the weakening of the shoots, particularly leaves, that had shown symptoms like a nutrient deficiency. Yellowing, and in some cases, tubular and green dried leaves were the most eminent symptoms of *Phomopsis* infection on the acacia. These symptoms were the result of necrosis and vascular wilt that were visible as necrosis of vascular under the bark of the branches (Fig. 5). These symptoms were different from the symptoms of the other three hosts and thus species identified on acacia (*P. theicola*) were different from the species identified on other hosts. The symptoms of *Phomopsis* on purple bauhinia were small round or oval spots and somewhat ingrown so that later a white to gray layer of mycelia covered the patch (Fig 5). These symptoms were similar to those in velvetleaf and hollyhock. However, the species identified from the purple bauhinia isolates were different from *P. loropetali*.

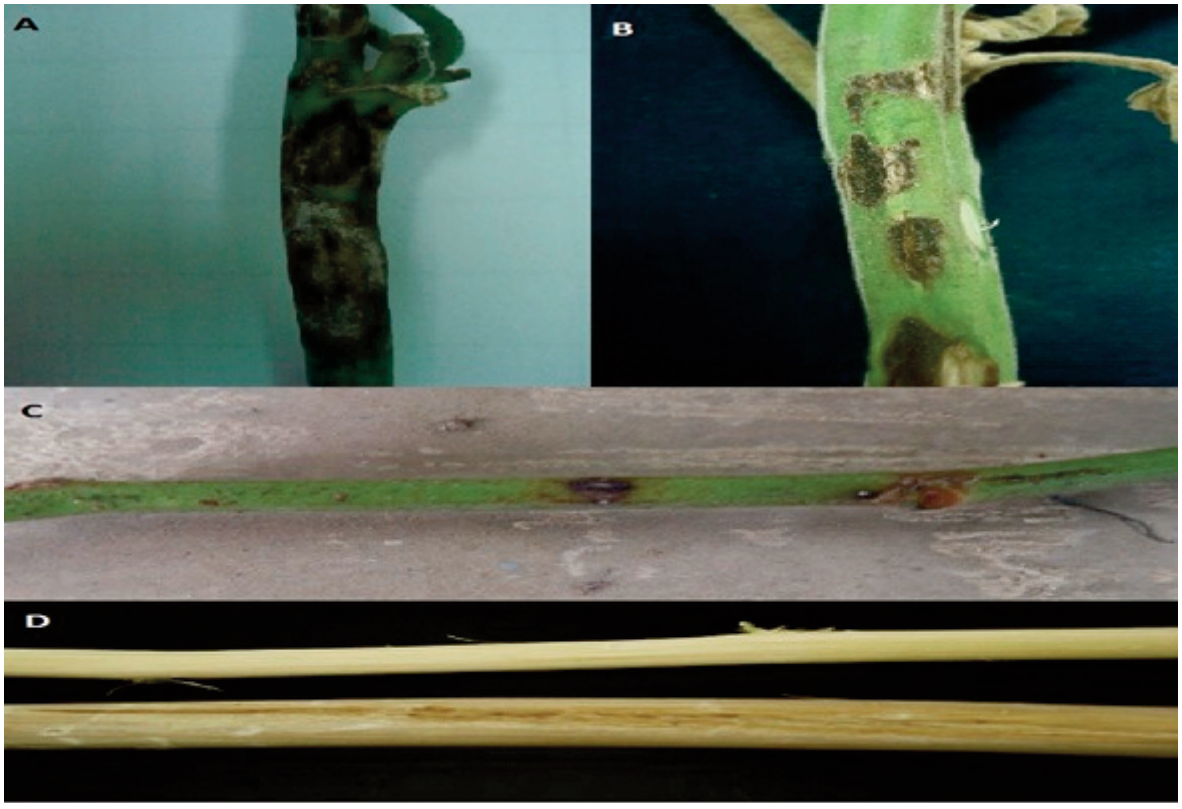

Fig. 5. Symptoms of the disease caused by the fungus *Phomopsis* on; A) hollyhock, B) velvetleaf, C) purple bauhinia, and D) acacia (the top branches are healthy and the bottom branches are infected).

DISCUSSION

The result of the nucleotide comparison showed that the isolates P1, P2, and P4 had the highest similarity with the species *Phomopsis malvacearum*. These results were consistent with the specifications of the morphology of isolates on velvetleaf and hollyhock, and they were iden-

tified as *P. malvacearum*. Mousavi *et al*. (2012) reported the species of *Phomopsis longicola* as the stem canker of velvetleaf plant. However, in this study, the velvetleaf isolates were diagnosed as *P. malvacearum*. On the other hand, Babaeizad and Sayari (2012) introduced *P. malvacearum* as the cause of canker in velvetleaf, which is in line with the results of this study. It seems that both species in the northern region of Iran can cause the canker of velvetleaf stem. The sequence of the P3 isolate showed the highest similarity with *Phomopsis loropetali*. This result confirmed the prominent difference among these species from other isolates in morphological studies. In fact, it had less growth in various growth media and smaller pycnidia than the other isolates. The sequence of the isolate P5 was significantly similar to the species *Phomopsis theicola*. This result is consistent with the morphological characteristics of isolates on acacia (oval pycnidia, larger than the other isolates) and the symptoms of the disease on acacia (necrosis and vascular wilt); the symptoms were not observed in the other three hosts. Therefore, the species identified on the acacia (*P. theicola*) was different from other host species. In this study, the amplification and sequencing of the TEF area were not suitable for identifying all *Phomopsis* isolates due to the lack of sufficient frequency related to the genus *Phomopsis* in GeneBank. Nevertheless, comparing sequences showed that the sequences of the isolates P1, P2, and P4 had the highest similarity with each other. Three studied isolates showed high similarity with each other, and, compared to the other sequences in the GeneBank, they were more similar to the *Phomopsis malvacearum* species. However, it seems that the TEF gene nucleotide sequence is not suitable for separating different species in the genus *Phomopsis*. The investigation of the type species of *Diaporthe* is recommended to find the relationship and the position of each species with its anamorph.

CONCLUSION

In total, the results of this study showed that *Phomopsis* is the cause of disease on four studied plants in the north of Iran and the causal fungus species are *Phomopsis malvacearum* on hollyhock (*Alcea rosea*), velvetleaf (*Abutilon theophrasti*), and one isolate of acacia (*Robinia pseudoacacia*)—*Phomopsis loropetali* on purple bauhinia (*Phanera purpurea*) and *Phomopsis theicola* on acacia (*Robinia pseudoacacia*).

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